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Evaluating the potential of marine *Bacteriovorax* sp. DA5 as a biocontrol agent against vibriosis in *Litopenaeus vannamei* larvae

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ABSTRACT

The potential application of *Bdellovibrio*-and-like organisms (BALOs) for the biocontrol of bacterial diseases has been widely recognized. However, no marine BALOs have been reported for *Vibrio*-related infections in penaeid shrimp. In the present study, the bacteriolytic ability of the marine *Bacteriovorax* strain DA5 against *Vibrio alginolyticus* zouA was examined by cocultivation and electron microscopy, and optimal lysis was observed at 30–35 °C and 20–30‰ salinity along with a high multiplicity of infection. Then, we showed that experimentally infected *Litopenaeus vannamei* larvae exhibited significantly higher survival with incremental DA5 levels. Finally, variation in the bacterial counts and the bacterial community in larval rearing water was investigated after prophylactic application of DA5. The elimination effect of DA5 on vibrios was visible at early time points, whereas only a few non-dominant bacteria, rather than the predominant populations, were affected through analysis of denaturing gradient gel electrophoresis of the 16S rDNA V3 region. Accordingly, the prophylactic and therapeutic efficacies of DA5 on vibriosis associated with *L. vannamei* could markedly enhance larval survivability, and these results will facilitate the application of marine *Bacteriovorax* to control vibriosis in shrimp larviculture.

1. Introduction

Shrimp aquaculture is widespread throughout tropical coastal areas and provides high-quality seafood for human consumption. However, this industry is beset by diseases mostly due to bacteria and viruses. For example, the Pacific white shrimp, *Litopenaeus vannamei*, one of the most widely cultured shrimp species, frequently suffers from *Vibrio*-related diseases when cultivated in shrimp hatcheries or in grow-out ponds (Lightner and Redman, 1998; Aguirre-Guzmán et al., 2001; Wen et al., 2008). During the larval nursery period, the eco-equilibrium of this artificial, closed, rearing water system, which has a high density of larvae, few to zero water exchange, is easily disrupted owing to the rapid proliferation of bacteria, especially certain vibrios. The major opportunistic pathogens in shrimp are *Vibrio harveyi*, *Vibrio alginolyticus*, and *Vibrio parahaemolyticus* (Lightner and Redman, 1998; Aguirre-Guzmán et al., 2001; Austin, 2010). Therefore, how to avoid or reduce mass larval mortality caused by vibriosis is still a

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key problem for the shrimp industry. In most cases, antibiotics are used routinely to prevent or treat vibriosis in shrimp, even though their side effects are well known. Although probiotics have been suggested as an alternative to antibiotics to control bacterial infections; however, many details await exploration (Balcázar et al., 2006; Defoirdt et al., 2007).

Bdellovibrio-and-like organisms (BALOs) are typically tiny, vibrioid-shaped bacteria with a single polar flagellum which replicate by predation on a wide variety of other susceptible Gram-negative bacteria (Jurkevitch and Davidov, 2007). BALOs have been isolated or recovered from various terrestrial or aquatic ecosystems, and they exhibit extensive diversity (Jurkevitch and Davidov, 2007). Generally, the strains isolated from marine or saltwater environments are defined as marine/halophilic BALOs, and are differentiated from freshwater/terrestrial BALOs mainly by their salt tolerance, G + C ratio, prey range, and habitat (Williams and Piñeiro, 2007). Phylogenetic analysis based on their 16S rDNA sequences demonstrates that almost all marine/halophilic BALO isolates are clustered in the genus *Bacteriovorax* of the family Bacteriovoracaceae (Baer et al., 2004; Pineiro et al., 2007; Wen et al., 2009a). Owing to their unique bactericidal ability, BALOs have the potential as biocontrol agents or living antibiotics against harmful bacteria (Sockett and Lambert, 2004), especially multidrug-resistant pathogens associated with infections in humans, animals, and plants (Dwidar et al., 2012). The use of freshwater/terrestrial BALOs to control bacterial infections has been shown to be effective for rabbit keratoconjunctivitis induced by *Shigella flexneri* (Nakamura, 1972), rice bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* (Nayak et al., 2002) and fish diseases caused by *Aeromonas hydrophila* (Chu and Zhu, 2010; Cao et al., 2012).

Diverse pathogens and opportunistic pathogens in mariculture are Gram-negative bacteria, and marine/halophilic BALOs have the ability to lyse most of them, especially those in the genus *Vibrio*, which are frequently reported as the preferred prey (Williams and Piñeiro, 2007) and may be naturally controlled due to predation of marine/halophilic BALOs (Richards et al., 2012). In addition, the structure and dynamics of the bacterial community may also be affected by BALOs predation (Rice et al., 1998; Davidov and Jurkevitch, 2004). However, little information could be obtained regarding to application of marine/halophilic BALOs as therapeutic agents to organisms that suffer from bacterial infections.

In this study, the lysis ability of a marine *Bacteriovorax* strain DA5 against a pathogen was initially investigated. Then, the therapeutic effect of DA5 on infected *L. vannamei* larvae and the prophylaxis of DA5 against vibriosis in shrimp larviculture were evaluated.

2. Materials and methods

2.1. Bacterial strains

Bacteriovorax sp. DA5 (DA5) was isolated and identified as a marine BALO in cluster X (Wen et al., 2009a), having the ability to lyse multiple *Vibrio* species (Wen et al., 2009b). *V. alginolyticus* zouA (zouA), identified as a pathogenic *Vibrio* strain isolated from diseased *L. vannamei* larvae by our group (Wen et al., 2008), was used both as prey for DA5 and as pathogen to challenge shrimp larvae. The cultivation and preparation of DA5 and zouA cells were performed according to the methods of Wen et al. (2009a).

2.2. Shrimp larvae and feed

L. vannamei larvae at nauplius stage 3–4 (N_{3-4}) were hatched from the same oosperm batch of a specific-pathogen-free broodstock in a commercial hatchery and were acclimatized in sterilized seawater before the experiments. Once larvae developed to N_6 stage, spirulina powder (TZU Feng Aquacultural Supplies Co. Ltd., Taiwan) and shrimp flakes (Yuh-huei Enterprise Co. Ltd., Taiwan) were used to feed larvae six times daily with levels of 0.15 and 0.5 mg L^{-1}, respectively, the levels of two diets were doubled once larvae developed to zoea 2 stage (Z_2), and then maintained until the end of the experiments.

2.3. Coculture of DA5 with zouA

To evaluate the predation kinetics of DA5 on zouA, these two organisms were cocultured in triplicate in 500-mL Erlenmeyer flasks containing 100 mL of seawater (salinity 30‰, pH 8.0) at 30 °C at 200 rpm for one week. The initial multiplicity of infection (MOI) of DA5 ($\sim 1 \times 10^7$ PFU mL^{-1}): zouA ($\sim 5 \times 10^8$ CFU mL^{-1}) was 1:50. Two types of controls, containing zouA (5×10^8 CFU mL^{-1}) only and DA5 (1×10^7 PFU mL^{-1}) only, were incubated synchronously with the same conditions. Then 1-mL water was sampled at 0, 3, 6, 9, 12, 15, 24, 48, 72, and 168 h for dilution plate counting of zouA and DA5. Thiosulfate citrate bile salt sucrose agar (TCBS; Beijing Land Bridge Technology Co. Ltd., China) was used to enumerate zouA. And seawater double-layer agar was adopted to quantify DA5 according to the method of Wen et al. (2009a) for examination of marine BALOs. In addition, for transmission electron microscopy, one drop of fresh DA5 lysate was sampled from the coculture at 12 h, and was placed on a Formvar film-coated copper grid (200 mesh) for 10 min, and then the excess liquid was removed by blotting. The sample was then counter-stained with a 2% (w/v) solution of phosphotungstic acid for 1 min and examined with a transmission electron microscope (JEM-2010 HR; JEOL, Japan).

To assess the optimal ranges of temperature, salinity, aeration, and MOI of DA5-mediated zouA lysis, the coculture described above was modified as follows. For temperature test, the cocultures were individually incubated at 20 °C, 25 °C, 30 °C, and 35 °C. For salinity test, the salinities of the cocultures were separately adjusted to 10‰, 20‰, 30‰, and 40‰ by addition of sea salt (Hailong, Xuwen Halobios Preparation Plant, China) dissolved in distilled water. Since the degree of aeration can be altered by changing the liquid volume in the Erlenmeyer flask, the four coculture volumes were set at 50, 100, 150, and 200 mL. To investigate the effect of MOI, the inoculum size of DA5 was changed, and the four initial ratios of DA5 to zouA were set at 1:10, 1:50, 1:100, and 1:1000. During the